Editorial

Stem Cell INVESTIGATION

Genome editing in human pluripotent stem cells: a systematic approach unrevealing pancreas development and disease

Elena Garreta[1], Andres Marco[1], Juan Carlos Izpisua Belmonte[2], Nuria Montserrat[1,3]

[1]Pluripotent Stem Cells and Activation of Endogenous Tissue Programs for Organ Regeneration (PR Lab), Institute for Bioengineering of Catalonia (IBEC), Barcelona, Spain; [2]Gene Expression Laboratory, Salk Institute for Biological Studies, La Jolla, CA 92037, USA; [3]Networking Biomedical Research Center in Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Madrid, Spain

Correspondence to: Nuria Montserrat, PhD. Pluripotent Stem Cells and Activation of Endogenous Tissue Programs for Organ Regeneration (PR Lab), Institute for Bioengineering of Catalonia (IBEC), Baldiri Reixac 15-21, 08028 – Barcelona, Spain. Email: nmontserrat@ibecbarcelona.eu.

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Although mouse models have represented a major tool for understanding and predicting molecular mechanisms responsible for several human genetic diseases, still species-specific differences between mouse and humans in their biochemical and physiological characteristics represent a major hurdle when translating promising findings into the human setting (1). For instance, in several types of maturity onset diabetes of the young (MODY; autosomal dominant), mice with heterozygous mutations do not develop diabetes (2). In this regard, the derivation of human embryonic stem cells (hESCs) in 1998 represented an unprecedented opportunity for human disease modelling, and a promising source for cell replacement therapies (3). Later on, the possibility to generate patient-derived induced pluripotent stem cells (iPSCs) has opened new venues for the potential translation of stem-cell related studies into the clinic (4).

Diabetes is one of the most common metabolic disorders worldwide, and a major cause of mortality and morbidity for which a cure remains elusive. Since the absence of functional insulin-secreting pancreatic β-cells results in diabetes, the possibility to generate functional β-cells from human pluripotent stem cells (hPSCs) has represented a major challenge in the field. Contrary to other prevalent disorders requiring different cell types in order to restore the loss of function in the damaged tissue (i.e., heart), β-cells derived from hPSCs would represent the only cell type missing in diabetes that could further be transplanted in non-endogenous sites, thus, representing a promising treatment for type 1 diabetics in the future (5).

Recent insights into β-cells derivation from hPSCs have contributed to the identification of transcriptional regulators and cell culture conditions converting terminally differentiated cells into β-cells, and even to define novel conditions sustaining β-cells replication *in vitro* and *in vivo* (6-8). Although these findings are encouraging, still the developmental mechanisms responsible of early and later stages of β-cell differentiation remain unclear. In addition, and more importantly, how these processes interfere in the acquisition of functional capabilities of β-cells after birth is still unknown.

In the work by Zhu *et al.* (9), the authors are able to examine these relevant questions in a controlled and unbiased manner. To this end, they systematically analyze the role of pancreatic lineage determinants in differentiation and disease making use of genome editing technology in hPSCs. In order to establish a cellular system for the interrogation of the putative role of specific factors with a known role in pancreas development in the murine system, the authors first generated a cellular platform for inducible gene expression for gain of function analysis in hESCs. For this purpose, the authors simultaneously integrated a constitutive promoter driving the expression of an optimized form of reverse tetracycline-controlled transactivator (M2rtTA), and a tetracycline-response element (TRE) driving the expression of the gene of interest (NotchIC and NGN3) in the *AAVS1* transgene safe harbor locus in the hESCs-HUES8 line by TALENs mediated gene editing. Next, in order to model human pancreatic development, the authors adapted an existing protocol

for direct pancreatic differentiation from hPSCs (10) and proceeded to characterize the different pancreatic populations emerged during the onset of differentiation using untargeted HUES8-hESCs. By this approach Zhu *et al.* derived definitive endoderm (DE) cells (expressing SOX17 and FOXA2); pancreatic progenitors (PP) expressing PDX1 (PDX1+); and polyhormonal β-cells (PH-β) expressing endocrine hormones characteristic of β and α cells. This model allowed the further analysis on the effect of *NGN3* and NOTCH perturbation in HUES8-hESC transgenic lines by the inducible expression of NGN3 (iNGN3) and *NotchIC* (iNothIC), revealing a conserved role of these factors between human and murine systems in pancreatic differentiation.

Next the authors interrogated the specific role of eight pancreatic transcription factors (PDX1, RFX6, PITF1A, GLIS3, MNX1, NGN3, HES1 and ARX) by combining TALEN and CRISPR/Cas-mediated gene editing in hPSCs. Six out of the eight factors are associated with permanent neonatal diabetes mellitus (PNDM), and biallelic inactivation of these genes is thought to be responsible for the absence of pancreatic endocrine cells in patients (PDX1, RFX6, PITF1A, GLIS3, MNX1, NGN3) (11-16). Similarly, mutations in both PDX1 and PITF1A were previously associated with pancreatic agenesis. In order to generate a massive platform allowing loss-of-function studies for the examination of the selected factors' role, the authors used a previously developed gene editing platform in hPSCs allowing doxycycline-regulated expression of the RNA-guided DNA endonuclease Cas9 (17). Using this system, simple transfection of synthetic chimeric guide RNAs (gRNAs) in doxycycline-treated hPSCs, allowed efficient generation of mutant hPSCs lines (17). In the current work, the authors increased the throughput of their platform designing two distinct gRNAs for each gene of interest that were synthesized in a compatible multi-well format, thus minimizing potential CRISPR/Cas9 off-target effects. In this manner, they were able to generate either biallelic "–/–" or monoallelic "–/+" knockout alleles carrying frameshift mutations. Of note, none of the studied mutations had an impact in the formation of DE. On the contrary, at the PP stage, RFX6$^{-/-}$ mutants showed a ~40% reduction of PDX1^{+} cells that was unrelated to a reduction in proliferation or an increase in apoptosis. Based on these findings, Zhu's conclusions were that RFX6 regulates PDX1 expression in a direct or indirect manner, and that the absence of RFX6 impairs the formation of PP cells. These results were in agreement with previous observations in Rfx6$^{-/-}$ mice (15) and patients carrying biallelic mutations in RFX6 (18), leading the authors to speculate that similar phenotype should be present during mice development.

Moreover, when the authors analyze PDX1$^{+/-}$ mutants they observed a reduction in the number of pancreatic endocrine cells expressing insulin and glucagon (characteristic of β and α cells, respectively). In order to prove that the observed phenotypes were due to haploinsufficiency and not to a possible dominant-negative effect, they derived biallelic mutant lines carrying the same mutations of the two heterozygous lines (either PDX1$^{L36fs/L36fs}$ or PDX1$^{L36fs/L34fs}$). Zhu *et al.* observed that no PDX1^{+} were derived in biallelic mutant lines, thus confirming that losing one functional PDX1 allele impairs pancreatic differentiation. These findings together with the fact that there is a clear association between PDX1 heterozygous mutations and genetic variants to type 2 diabetes (2,19,20) led the authors to conclude that defects in β cell development may predispose to diabetes.

Following with their systematic and accurate analysis, the authors also found out that contrary to *Ngn3*$^{-/-}$ mutant mice, where no INS^{+} cells are detected during the onset of pancreatic development, NGN3$^{-/-}$ hESC lines still gave rise to a small percentage of insulin positive cells (INS^{+}). Since a small fraction of patients diagnosed with permanent or transient neonatal diabetes carrying biallelic *NGN3* mutations also displayed low levels of blood C-peptide (12,21), the authors suggested that in NGN3$^{-/-}$ hESCs lines, β cells could still be formed in the absence of any NGN3 activity. In order to test this hypothesis, they generated another battery of NGN3 mutants (NGN3 null mutants and NGN3 disease-mimicking lines) that were INS^{+} at the PH-β stage. These observations were in agreement with findings in *NGN*-deficient patients that displayed C-peptide levels besides disease-associated symptoms. These findings lead the authors to interrogate until which extent NGN3$^{-/-}$ derived cells could give rise to mature β cells. To this end, Zhu *et al.* engineered 2 additional lines on the NGN3$^{-/-}$ background through homology-directed repair (HDR) using a single-stranded DNA donor (NGN3$^{Cr/Cr}$ lines). Then, NGN3$^{Cr/Cr}$ lines together with wild type counterparts were further differentiated to insulin secreting cells (β-like cells) following a previously reported protocol (5,22). By this elegant approach, the authors unambiguously showed that β-like cells derived from NGN3$^{-/-}$ lines that were positive for C-peptide expression (CPEP+; ~0.5%) did not co-express glucagon neither somatostatin, but NKX6.1 (~0.05% from the total population). Moreover, β-like

cells derived from NGN3$^{-/-}$ lines did not exhibit glucose-stimulated insulin secretion. All these results led the authors to conclude that NGN3 is not absolutely required for the formation of monohormonal CPEP+, but may lead to impaired β cell function in *NGN3*-deficient patients. Lastly, in order to determine the developmental window for NGN3 activity during human pancreas formation, the authors also generated inducible NGN3 lines in the NGN3$^{-/-}$ hESC background by replacing the Puro-iCas9 cassette targeted in the *AAVS1* locus with an hygro-iNGN3 transgene through HDR. In their hands, the expression of *NGN3* transgene gave rise to the generation of endocrine cells at all the evaluated stages, with major effects on the generation of both PDX1^{+}/NKX6.1^{-} and PDX1^{+}/NKX6.1^{+} β-cells.

Overall, the work led by Dr. Huangfu demonstrates the power of genome editing combined with hPSCs technology in order to systematically explore the role of a large number of candidate genes previously related with PNDM and pancreatic development in the human setting. Expanding the potential of their cellular platform by the generation of multiple cell lines by HDR, the authors developed a large number of mutant hPSCs in a short time period validating the observed cellular phenotypes at the mechanistic level. Importantly, the authors were also able to identify previously unknown effects when mutating pancreatic transcription factors related with PDNM, as RFX6, identified in this work as a key factor necessary for both early formation of PP and the development of functional endocrine cells. Overall, Zhu *et al.* are to be congratulated for adding a comprehensive view about genome editing possibilities when modeling human differentiation and disease. This work highlights the use of this powerful cellular toolbox for the validation of *in vivo* studies avoiding confounding effects related to the limitations of murine models or other issues related with the use of patient derived iPSCs for disease modeling (i.e., need of primary patient samples, differences in differentiation efficiencies, among others) (23).

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Footnote

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